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Anemia paripinnata (Anemiaceae), a New Species from Central Brazil

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ABSTRACT.—We describe *Anemia paripinnata*, a new species from the rock outcrops of Central Brazil. It is remarkably distinct from most of its congeners by having paripinnate lamina, a character that is only found in the Amazonian *A. dardanoi*. The new species is described, illustrated, and compared to the most similar species in the genus. The new species is known from few collections in Mato Grosso. Based on the IUCN criteria, it can be considered an endangered species.

KEY WORDS.—Biodiversity; Brazilian Savannah; Cerrado; Ferns; Taxonomy

Anemia Sw. belongs to the family Anemiaceae, and comprises about 115 species (Mickel, 2016). Phylogenetic studies have supported *Anemia* as monophyletic (Skog, Zimmer and Mickel, 2002; Wikström, Kenrick, and Vogel, 2002; Labiak, Mickel, and Hanks, 2015), with the often segregated genus *Mohria* Sw. nested within it. Based on these relationships, Mickel (2016) recognized three subgenera in *Anemia*: *Anemia*, *Mohria* (Sw.) Mickel, and *Anemiorrhiza* Prantl. The genus is characterized morphologically by having sporangia with a complete apical annulus and spores that are generally striate and tetrahedral (Mickel, 1962; Labiak, Mickel, and Hanks, 2015). In most of its species, the sporangia are limited to the basal pair of pinnae, which are erect and have greatly reduced laminar tissue. Regarding the geographic distribution, subgen. *Anemia* has the widest distribution, occurring in the Neotropics, Africa, Madagascar, and India. On the other hand, subgen. *Mohria* is restricted to Africa, and subgen. *Anemiorrhiza* is found only in Central America and the Antilles, with a strong preference for limestone formations (Labiak, Mickel, and Hanks, 2015; Mickel, 2016).

Different from most ferns, *Anemia* is commonly found in open and dry areas, growing both as terrestrial or epipetric – no epiphytes are known in the genus (Labiak, Mickel, and Hanks, 2015; Mickel, 2016). The genus is also well known

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for having many hybrids and polyploids, and apparently the great majority of species are of hybrid origin (Mickel, 1962, 1982).

Brazil is the main center of diversity for *Anemia*, with ca. 60 species. In Brazil, the Central Plateau is one of the most important areas of endemism for *Anemia*, where about 30 species can be found (Mickel, 2016). Although several species have been recently described from these rock outcrops (Mickel, 2016), new collections from the state of Mato Grosso revealed a new species, which we describe herein.

MATERIAL AND METHODS

Specimens were studied at MBM, RB, and SP. In the descriptions, the largest leaf on each specimen was measured for length and width. In order to examine the perine ornamentation, spores were transferred with dissecting needles from herbarium specimens to aluminum stubs coated with asphalt adhesive. The stubs were then sputter-coated with gold for 2.5 min, and spores were imaged digitally using a JEOL JSM-6360LV scanning electron microscope at the Electron Microscopy Center of Universidade Federal do Paraná, Brazil. Coordinates were converted to Decimal Degrees using the on-line tool available at <http://splink.cria.org.br/conversor>, and are presented in brackets. The dot-distribution map was compiled from all specimens studied and generated with QGIS version 2.0.1 (Quantum GIS Development Team, 2013). Layers included a delimited text file compiled from all specimens studied, a raster file (1:10,000,000) from Natural Earth (www.naturalearthdata.com), and shape files (political units and rivers) obtained from the Organization for Flora Neotropica (www.nybgpress.org). Conservation Status assessment was made using GeoCAT (online tool available at <http://geocat.kew.org/editor>) (Bachman *et al.*, 2011; Bachman and Moat, 2012).

RESULTS AND DISCUSSION

Anemia paripinnata Labiak & Mickel, **sp. nov.** TYPE: BRAZIL. Mato Grosso: Santa Cruz do Xingu. Parque Estadual do Xingu, limite norte do Parque, 09°45'10"S [-9.752778], 52°37'56"W [-52.632222], 277 m, 10 Mar 2011, D. C. Zappi, W. Milliken, S. Frisby, C. R. A. Soares, A. F. Forte, D. R. Silva & L. F. Lindolfo, 3275 (Holotype: RB!; Isotypes: K, SP!, UNEMAT). (Fig. 1.)

Diagnosis.—This species is similar to *Anemia dardanoi* Brade by having paripinnate laminae. It differs from *Anemia dardanoi* by having coriaceous laminae, linear-elliptic pinnae, and by its spores that are 55–60 µm diam, and striae that are half of the ridge width.

Plants epipetric or terrestrial. **Rhizomes** horizontal, short-creeping, compact, approximately 0.5 cm diam.; **rhizome hairs** orange to orange-brown, 1.5–3 mm long, 4–7 celled. **Fronds** hemidimorphic; sterile fronds 8–15 cm long, fertile fronds 20–30 cm long; **petiole** 2–4(5) times longer than the lamina in the sterile

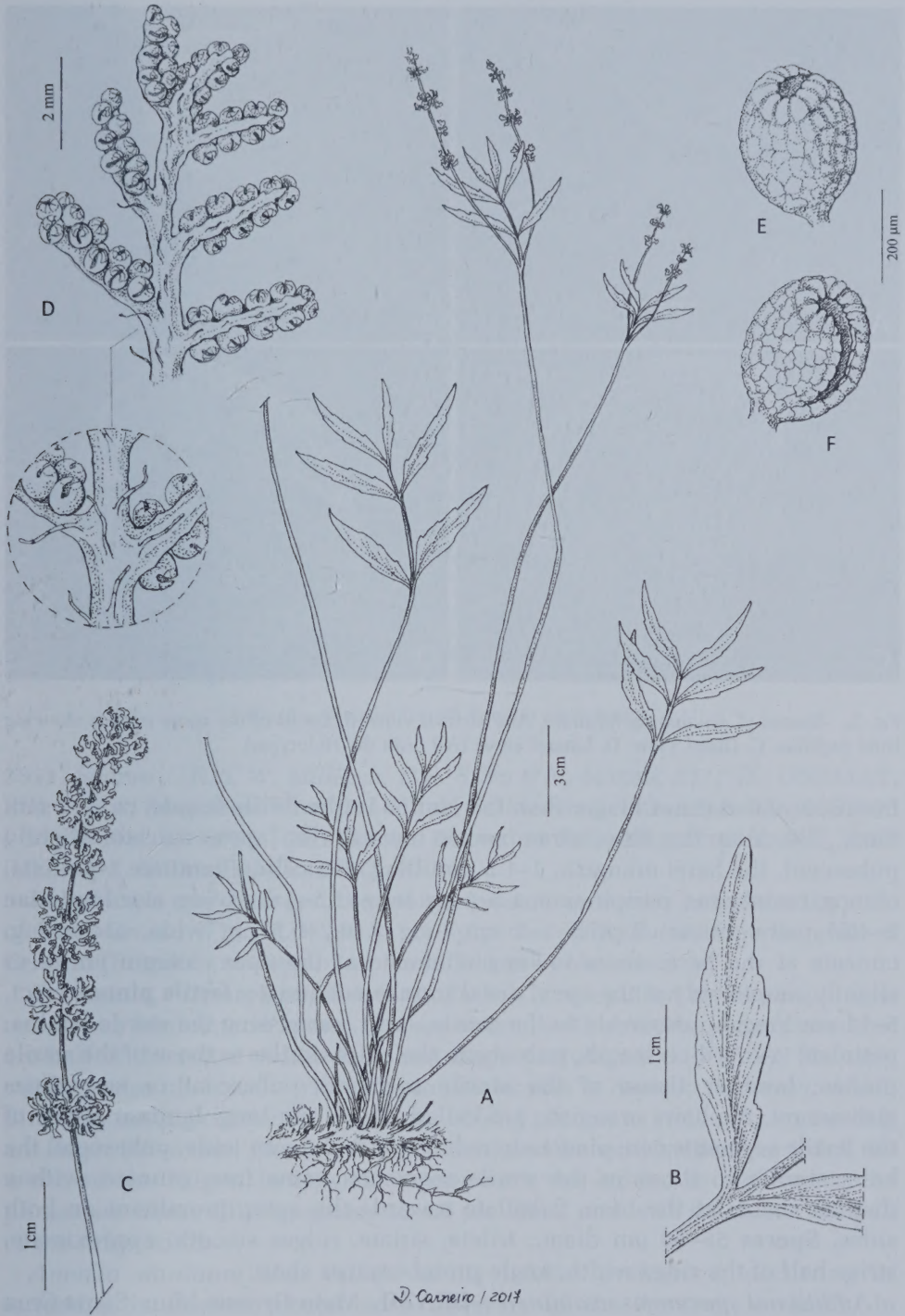


FIG. 1. *Anemia paripinnata*. A. Habit. B. Detail of a pinna. C. Fertile pinna. D. Detail of a fertile pinna showing the reduced laminar tissue. E. Sporangium with apical annulus. F. Opened sporangium. (All from the Holotype).

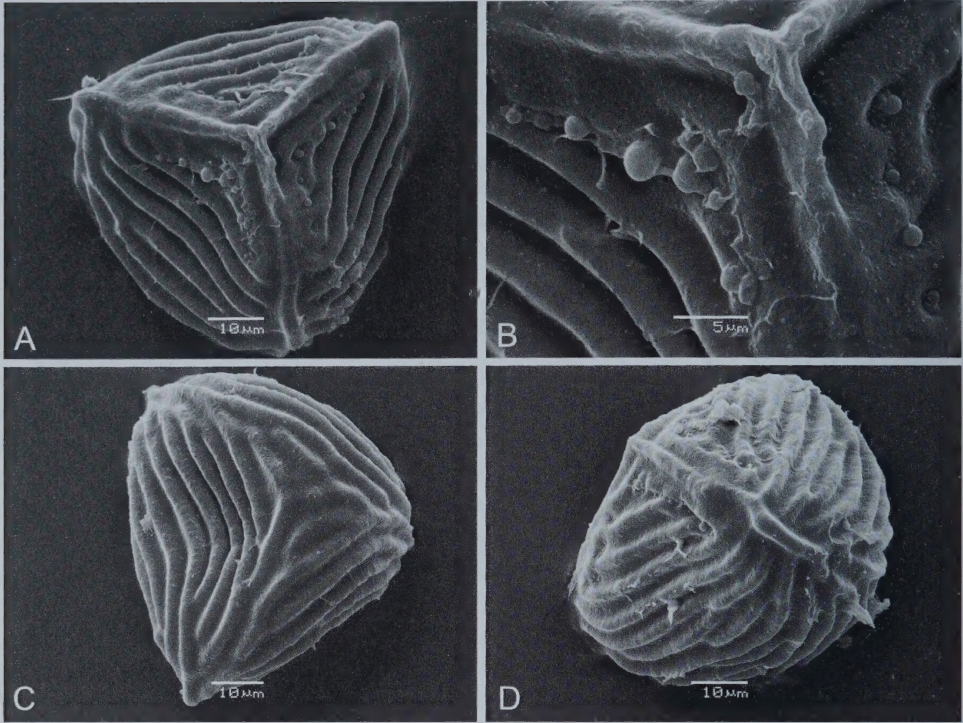


FIG. 2. Spores of *Anemia paripinnata*. A. Proximal view. B. Detail of the spore surface showing little papillae. C. Distal View. D. Lateral view. (All from the Holotype.)

fronds, and 6–8 times longer than the lamina in the fertile fronds, ca. 1–2 mm diam., black at the base, stramineous towards the apex, sulcate, slightly pubescent, the hairs orangish, 1–1.5 mm long, 3–5 celled; **laminae** 1-pinnate, oblong, coriaceous, paripinnate, 1.5–7 cm long \times 1.5–5 cm wide; **sterile pinnae** 2–4(5) pairs, linear-elliptic, 1–2 cm long \times 0.2–0.5 cm wide, ascending, cuneate at the base, acute to long attenuate at the apex, margin entire or slightly serrate only at the apex, distal pinnae not adnate; **fertile pinnae** erect, 5–11 cm long, approximate to the sterile, erect, surpassing the sterile lamina, petiolate $\frac{1}{2}$ – $\frac{3}{4}$ their length, pubescent, the hairs similar to those of the sterile pinnae; **laminar tissue of the sterile segments** pubescent or sometimes glabrescent, the hairs orangish, 3–5 celled, 1–1.5 mm long; **laminar tissue of the fertile segments** conspicuously reduced, 0.3–0.5 mm wide, pubescent, the hairs similar to those in the sterile segments; **veins** free, pinnate, with a distinct costae at the base, flabellate towards the apex, prominent on both sides. **Spores** 55–60 μ m diam., trilete, striate, ridges smooth, approximate, striae half of the ridge width, angle protuberances short.

Additional specimens examined.—**BRAZIL.** Mato Grosso: Mun. Santa Cruz do Xingu. Parque Estadual do Xingu, limite nordeste do Parque, subindo o Rio Fontourinha. 09°43'4"S [-9.717778], 52°22'12"W [-52.370000], 280 m, 01 Mar



FIG. 3. Distribution of *Anemia paripinnata*.

2011, Soares, C.R.A, W. Milliken, D.R. Silva & V. Marini, 3117 (K, UNEMAT, RB); Itaúba, Resgate de Flora da UHE de Colíder, Lote E de supressão, 0653506 [-55.595095], 8786738 [-10.972183], 281 m, 20 Feb. 2015, Engels, M.E. 2768 (HERBAM, MBM, TANG, CNMT, RB).

Distribution and ecology. *Anemia paripinnata* is known only from the state of Mato Grosso (Fig. 3). It grows as epipetric on rock outcrops in areas of Cerrado (Brazilian savannah), at about 280 m in elevation.

Comments. *Anemia paripinnata* can be easily recognized by its paripinnate, coriaceous lamina, and spores that are striate, with smooth ridges, striae that are half of the ridge width, and short angle protuberances (Fig. 2). The only other species of *Anemia* with paripinnate lamina is *Anemia dardanoi*, which differs by having chartaceous laminae, oblong pinnae, as well as by spores that are larger (66–81 μm , 74 μm average diam.) and have striae that are twice the width of the ridges. In *Anemia paripinnata* the lamina is coriaceous, the pinnae are linear-elliptic, spores 55–60 μm diam. and with striae that are half of the ridge width.

Anemia dardanoi also differs in its habitat preference, occurring as terrestrial in shaded places close to rivers in the Amazon lowlands. In contrast, *A. paripinnata* occurs as epipetric on rock outcrops, or as terrestrial in open sandy areas.

Mickel (2016) considers the absence of a terminal pinna in *A. dardanoi* to be most likely an aberration. Although we have seen only three collections of *A. paripinnata*, there are at least a dozen individuals, all with paripinnate laminae. Therefore, we consider it to be a normal condition for this species, instead of an aberration as proposed by Mickel (2016) for *A. dardanoi*.

Based on the characteristics of its spores (striate, ridges smooth, with striae half of the ridge width, and angle protuberances short), coriaceous laminae, as well as prominent veins, *Anemia paripinnata* appears to be related to the *Anemia rutifolia* clade, as proposed by Labiak, Mickel, and Hanks (2015). However, its phylogenetic relationships remain to be tested.

Conservation status. *Anemia paripinnata* is currently known only from three collections: two of them from the same locality (Parque Estadual do Xingu), and one collected in an area where there is now a hydroelectric plant. Based on our current knowledge on this species, GeoCAT suggests that it has an extent of occurrence of 1,345,433 km² and an area of occupancy of approximately 12,000 km². According to these data and the IUCN Red List criteria (IUCN 2012), it is assessed here as an endangered species (EN).

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×*Lindsaeosoria flynnii* (Lindsaeaceae), Another Confirmed Example of Deep Hybridization Among the Ferns

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ABSTRACT.—The origin of ×*Lindsaeosoria flynnii* as an intergeneric hybrid between *Lindsaea ensifolia* and *Odontosoria chinensis* is here confirmed by molecular analysis of plastid data and Illumina high-throughput sequencing of the low-copy nuclear *LEAFY* gene. Plastid DNA grouped the hybrid together with *Lindsaea ensifolia*, suggesting that it is the maternal progenitor, while the presence of distinct *LEAFY* alleles confirmed the hybrid origin. Together with ×*Cystocarpium* this is confirmed example of deep hybridization event between fern lineages that were separated in the early Cenozoic or late Mesozoic.

KEY WORDS.—cpDNA, deep hybrid, *LEAFY*, *Lindsaea*, *Odontosoria*

Hybridization is widespread among the ferns and by reorganizing genomes it may play a significant role in driving fern evolution and diversity dynamics (Sigel, 2016). In addition to numerous interspecific fern hybrids and hybrid species complexes, a large number of intergeneric hybridization events have been postulated (Table 1). However, most of the proposed fern nothogenera represent congeneric hybridization events under current generic classifications (Christenhusz, Zhang, and Schneider, 2010; PPG I, 2016). Although many of the presumed hybrids may not be as dramatic crosses as originally thought, there is compelling molecular evidence for a truly deep intergeneric hybrid origin of ×*Cystocarpium* Fraser-Jenk. as a cross between *Cystopteris* Bernh. and *Gymnocarpium* Newman, which are estimated to have diverged ca. 60 million years ago (Rothfels *et al.*, 2015). At least three other nothogenera are postulated as hybrids between fern genera with comparable divergence time estimates, namely ×*Dryostichum* W.H. Wagner and ×*Polysticalpe* Fraser-Jenk. as crosses between *Dryopteris* Adans. and *Polystichum* Roth, and ×*Lindsaeosoria* W.H. Wagner as a cross between *Lindsaea* Dryand. ex Sm. and *Odontosoria* Fée.

×*Lindsaeosoria flynnii* W.H. Wagner was found in Hawaii (Kauai) in 1987 (Wagner, 1993). The hybrid was presumed to have originated as an intergeneric cross between *Odontosoria chinensis* (L.) J. Sm., a species native to Hawaii, and *Lindsaea ensifolia* Sw., a locally naturalized species (Vernon and Ranker, 2013; Wagner, 1993). Both parental genera belong to the family Lindsaeaceae (PPG I, 2016), which is among the “early-diverging” Polypodiales in the fern phylogeny (Lehtonen, 2011; PPG I, 2016). Under some analytical settings the presumed parental genera were resolved as sister lineages, but most analyses have resolved them as more distant relatives (Lehtonen *et al.*, 2010; Lehtonen, Wahlberg, and Christenhusz, 2012; Regalado *et al.*, 2017). Morphologically, the presumed parental species are highly

TABLE 1. Fern nothogenera with notes on their current taxonomic status.

Family	Nothogenus	Presumed parentage	Notes*
Aspleniaceae	× <i>Asplenoceterach</i> D.E. Mey. × <i>Asplenophyllitis</i> Alston × <i>Asplenosorus</i> Wherry × <i>Ceterophyllitis</i> Pic.Serm. × <i>Asplenicystopteris</i> P. Fourn.	<i>Asplenium</i> L. × <i>Ceterach</i> Willd. <i>Asplenium</i> L. × <i>Phyllitis</i> Hill <i>Asplenium</i> L. × <i>Camptosorus</i> Link <i>Ceterach</i> Willd. × <i>Phyllitis</i> Hill <i>Asplenium</i> L. × <i>Cystopteris</i> Bernh.	<i>Ceterach</i> = <i>Asplenium</i> <i>Phyllitis</i> = <i>Asplenium</i> <i>Camptosorus</i> = <i>Asplenium</i> <i>Ceterach</i> and <i>Phyllitis</i> = <i>Asplenium</i> hybrid origin probably mistaken (Knobloch, Gibby and Fraser-Jenkins, 1984)
Aspleniaceae × Cystopteridaceae	× <i>Cornoathyrium</i> Nakaike × <i>Depazium</i> Nakaike × <i>Neotribleparia</i> Nakaike	<i>Cornopteris</i> Nakai × <i>Athyrium</i> Roth <i>Deparia</i> Hook. & Grev. × <i>Diplazium</i> Sw. <i>Neotriblemma</i> Nakaike × <i>Deparia</i> Hook. & Grev.	<i>Cornopteris</i> = <i>Athyrium</i> × <i>Depazium</i> = <i>Deparia</i> <i>Neotriblemma</i> = <i>Deparia</i> (Christenhusz, Zhang, and Schneider, 2010)
Cyatheaaceae	× <i>Cyathidaria</i> Caluff & Shelton × <i>Cystocarpium</i> Fraser-Jenk.	<i>Cyathea</i> Sm. × <i>Gnemidaria</i> C. Presl <i>Cystopteris</i> Bernh. × <i>Gymnocarpium</i> Newman	<i>Gnemidaria</i> = <i>Cyathea</i> Intergeneric hybrid origin confirmed (Rothfels et al., 2015)
Cystopteridaceae	× <i>Cyclobotrya</i> Engels & Canestraro × <i>Dryostichum</i> W.H. Wagner	<i>Cyclodium</i> C. Presl × <i>Polybotrya</i> Humb. & Bonpl. ex Willd. <i>Dryopteris</i> Adans. × <i>Polystichum</i> Roth	Presumed intergeneric hybrid (Engels and Canestraro, 2017) Presumed intergeneric hybrid (Wagner et al., 1992)
	× <i>Leptoarachniodes</i> Nakaike	<i>Leptorumohra</i> (H. Ito) H. Ito × <i>Arachniodes</i> Blume	<i>Leptorumohra</i> = <i>Arachniodes</i>
	× <i>Polysticalpe</i> Fraser-Jenk.	<i>Polystichum</i> Roth × <i>Diacalpe</i> Blume	<i>Diacalpe</i> = <i>Dryopteris</i> . Suggested intergeneric hybrid (Fraser-Jenkins, 1997)
Lindsaeaceae	× <i>Lindsaeosoria</i> W.H. Wagner	<i>Lindsaea</i> Dryand. ex Sm. × <i>Odontosoria</i> Fée	Intergeneric hybrid origin confirmed here
Polypodiaceae	× <i>Aglaonaria</i> Hoshiz. × <i>Pleopodium</i> Schelpe & N.C. Anthony	<i>Aglaomorpha</i> Schott × <i>Drynaria</i> J.Sm. <i>Pleopeltis</i> Humb. & Bonpl. ex Willd. × <i>Polypodium</i> L.	<i>Drynaria</i> = <i>Aglaomorpha</i> Current circumscription of the genus <i>Pleopeltis</i> includes parents of the hybrids (Smith and Tejero-Díez, 2014)

TABLE 1. Continued.

Family	Nothogenus	Presumed parentage	Notes*
Pteridaceae	\times <i>Coniodictyogramme</i> Nakai	<i>Coniogramme</i> Fée \times <i>Dictyogramme</i> Fée, nom. rej.	<i>Dictyogramme</i> = <i>Coniogramme</i>
	\times <i>Eriosonia</i> Pic.Ser.	<i>Eriosorus</i> Fée \times <i>Jamesonia</i> Hook. & Grev.	<i>Eriosorus</i> = <i>Jamesonia</i>
	\times <i>Hemionanthes</i> Mickel	<i>Hemionitis</i> L. \times <i>Cheilanthes</i> Sw.	Presumed intergeneric hybrid (Mickel, 1987) although generic affinities of putative parents uncertain (Windham <i>et al.</i> , 2009)
Tectariaceae	\times <i>Pityrogramma</i> L.D. Gómez	<i>Pityrogramma</i> Link \times <i>Trismeria</i> Fée	<i>Trismeria</i> = <i>Pityrogramma</i>
	\times <i>Pleuroderris</i> Maxon	<i>Tectaria</i> Cav. \times <i>Dictyoxiphium</i> Hook.	<i>Dictyoxiphium</i> = <i>Tectaria</i>
	\times <i>Chrimatopteris</i> Quansah & D.S. Edwards	<i>Christella</i> H. Lévl. \times <i>Pneumatopteris</i> Nakai	Generic delimitation of the parental genera remains uncertain
Thelypteridaceae	\times <i>Chrinephrium</i> Nakaike	<i>Christella</i> H. Lévl. \times <i>Pronephrium</i> C. Presl	Generic delimitation of the parental genera remains uncertain

* Classification follows PPG I (2016) unless otherwise noted.

distinct: *L. ensifolia* has erect, simply-pinnate coenosoral leaves with reticulate venation, in contrast to spreading, 3-pinnate or more divided leaves with free venation and discrete sori in *O. chinensis* (Kramer, 1971). Consequently, \times *Lindsaeosoria* has an unusual morphology combining characters of both presumed parents (Wagner, 1993). In addition to these three taxa *L. repens* (Bory) Thwaites var. *macraeana* (Hook. & Arn.) Mett. ex Kuhn is the only other Lindsaeaceae in Hawaii (Gustafson, Herbst, and Rundel, 2014): This taxon is morphologically very different and not closely related to *L. ensifolia* (Lehtonen *et al.*, 2010). The timing of divergence between *Lindsaea* and *Odontosoria* remains controversial with age estimates for the split ranging from Eocene to late Jurassic (Lehtonen, Wahlberg, and Christenhusz, 2012; Lehtonen *et al.*, 2017; Regalado *et al.*, 2017; Schuettelpelz and Pryer, 2009; Testo and Sundue, 2016).

The purpose of this study is to verify the presumed hybrid origin of \times *Lindsaeosoria* as a hybrid between *L. ensifolia* and *O. chinensis*, using plastid and nuclear DNA sequence data.

MATERIALS AND METHODS

Total genomic DNA was extracted from an herbarium specimen of \times *Lindsaeosoria flynnii* (Flynn 2351) deposited in the herbarium Z (herbarium acronyms according to Thiers, 2017) using E.Z.N.A. SP plant DNA kit (Omega Bio-tek, Doraville, Georgia) with a protocol of 30 min incubation, 600 μ l of cell lysis buffer, 50 μ l of elution buffer and 10 min elution time. Five plastid markers (*rpoC1* gene, *rps4* gene, *rps4-trnS* intergenic spacer, *trnL-trnF* intergenic spacer, *trnH-psbA* intergenic spacer) were amplified using PureTaq RTG PCR beads (Amersham Biosciences, Piscataway, New Jersey). Primers used for amplification and sequencing were: *trnL-trnF* primers e and f (Taberlet *et al.*, 1991); *trnH-psbA* primers trnH (Tate and Simpson, 2003) and psbA3'f (Sang, Crawford, and Stuessy, 1997); *rpoC1* primers LP1 and LP5 (Chase *et al.*, 2007); *rps4-trnS* primers trnSGGA (Shaw *et al.*, 2005) and rps4.5' (Small *et al.*, 2005). The amplification profiles were as in Lehtonen *et al.* (2010).

In order to amplify and sequence the low-copy nuclear gene *LEAFY* from the relatives of \times *Lindsaeosoria*, I used a set of primers (forward primer lfyEX-F1: AAG CGT CAA GAG GAG GAC AT located in the exon 1 and forward primers lfyF7: ACA AGG GAG CAT CCC TTC ATA G and lfyF6: AGG GAG CAA AGA GGG GAT AAT G located in the exon 2; reverse primers lfyR3: CTC AAC AGG TTG GAT TTC TCC and lfyR4: ATR CGG AGC TTC TCR TTC C located in the exon 3) designed on the basis of the *LEAFY* sequences available at GenBank and using Primer3 software (Untergasser *et al.*, 2012) to aid the design process. *LEAFY* was amplified using Phusion Flash PCR Master Mix (Finnzymes, Espoo, Finland) with an amplification profile of 10 s denaturation at 98°C followed by 30 cycles of 98°C for 1 s, 59–63°C (depending on the primers) for 5 s, 72°C for 20 s, and a final extension of 1 min at 72°C. The PCR products were purified and sequenced in both directions under BigDye™ terminator cycling conditions by Macrogen Inc., Seoul, South Korea/Amsterdam, the Netherlands

(www.macrogen.com). Traditional Sanger sequencing was applied without cloning for the putatively non-hybrid species and since the sequence chromatograms were generally clear and did not show polymorphic sites or high levels of noise, these sequences were deemed suitable for the purpose of the present study. However, sequencing of the first intron failed for most of the taxa and the final dataset is most complete for the second intron.

×Lindsaeosoria was sequenced differently. First, additional *LEAFY* forward (lfyNGSf: TCT ACG AGC AGT GTG CAA GG) and reverse (lfyNGSr: TTG GCA TGT CGA AAC ACT TG) primers were designed by comparing the sequences of *L. ensifolia* and *O. chinensis*. These primers were then used to amplify a ca. 400 bp region of the *LEAFY* second intron. The PCR product was then sequenced at Macrogen Inc. using a high-throughput Illumina HiSeq 2500 platform and 100 bp long paired-end reads. The raw sequence reads were cleaned by trimming adapter sequences and low-quality bases and filtering reads shorter than 90 bp in cutadapt (Martin, 2011). The cleaned sequence reads were deduplicated with Tally (Davis *et al.*, 2013) and only those unique reads were kept that had over 5% frequency of occurrence.

Amplification success of the *LEAFY* gene varied highly in the initial trials and therefore the final taxon sampling was limited to a set of species from which the amplicons were easily produced. The final data set included five species of *Lindsaea* (including *L. ensifolia*), *Nesolindsaea kirkii* (Baker) Lehtonen & Christenh., *Odontosoria chinensis* and *Saccoloma inaequale* (Kunze) Mett. as the outgroup. The Hawaiian *L. repens* var. *macraeana* was not included in the analysis but *Lindsaea* sp2 is the closest sampled relative of *L. repens* -group (Lehtonen *et al.*, 2010). Voucher information and GenBank accession codes are given in Appendix A.

Sequences produced with Sanger sequencing were aligned with default parameters in MAFFT v7.215 (Kato and Standley, 2013) and the deduplicated Illumina reads were aligned to this alignment by hand, without manipulating the original sequence alignment or deleting any portions of it. Appropriate evolutionary models were selected from among the three substitution schemes available in MrBayes v.3.2.2 (Ronquist *et al.*, 2012) on the basis of the Bayesian Information criterion using jModelTest 2.1.4 (Darriba *et al.*, 2012). The best-fitting models were HKY for *trnL-trnF*, HKY+I for *LEAFY* and *rpoC1*, HKY+G for *trnH-psbA*, GTR for *rps4-trnS*, and GTR+I for *rps4*. The plastid markers were concatenated since they were expected to share a common evolutionary history and phylogenies were inferred separately for the plastid and nuclear data. Length of the concatenated alignment of plastid markers was 2671 bp and the matrix had 2% of the bases missing. *LEAFY* matrix was 809 bp in length and it had 48% of the bases missing with most of the taxa missing the first intron. Bayesian searches were implemented in MrBayes v.3.2.2 (Ronquist *et al.*, 2012), using two independent runs and four chains for 10 million MCMC generations sampling every 1000 generations. All the model parameters were unlinked between the partitions and the default exponential branch length and uniform topology priors were applied. Convergence of the runs was visually confirmed in Tracer v1.6.0 (Rambaut *et al.*, 2013) and conservatively the first

25 % of the trees were discarded as burn-in. The effective sample sizes for all parameters in the merged runs were over 1000. Multiple sequence alignments as well as phylogenetic trees are available at TreeBASE (S21891).

RESULTS

The Illumina sequencing of *LEAFY* from $\times L.$ *flynnii* resulted in three different paired-end sequence pairs, each totaling 200 bp in length, with over 5% frequency of occurrence among all the reads. One of them perfectly matched with the sequence of *O. chinensis* while another one was identical to the sequence of *L. ensifolia*. The third one differed from the second one by a single substitution. *Odontosoria chinensis* and *L. ensifolia* differed from each other by 17 substitutions and three indels across the same alignment region.

The plastid and *LEAFY* trees obtained were generally congruent with each other and with the broader phylogenetic analyses (Lehtonen *et al.*, 2010), with the exception that *Odontosoria* and *Nesolindsaea* were grouped together in the plastid tree, albeit without support (Fig. 1). In the plastid tree $\times L.$ *Lindsaeosoria* was resolved as sister to *L. ensifolia*, but their plastid sequences were not identical (altogether five substitutions and one indel differentiated the two taxa). This result is not surprising, given that *L. ensifolia* is a taxonomically challenging species complex and the sample analyzed here originated from La Réunion. The *LEAFY* tree generally had low posterior probabilities, most likely as a result of limited available data. Despite of this, the topology was fully congruent with the current understanding of Lindsaeaceae phylogeny (Lehtonen *et al.*, 2010). Of the three *LEAFY* alleles sequenced from $\times L.$ *Lindsaeosoria* one was sister to *O. chinensis*, while the other two grouped with *L. ensifolia*.

DISCUSSION

This study provides molecular evidence that $\times L.$ *Lindsaeosoria* is indeed an intergeneric hybrid between *Lindsaea ensifolia* and *Odontosoria chinensis*. The parental species share a wide, largely sympatric paleotropical distribution (Kramer, 1971), but only eight hybrid individuals have been observed at one location in Kauai (Wagner, 1993). The hybridization event must have occurred recently, since *Lindsaea ensifolia* was collected for the first time in Hawaii in 1969 (Wagner, 1971) and the hybrids were observed in 1987 (Wagner, 1993). Plastid DNA is apparently maternally inherited in ferns (Gastony and Yatskievych 1992; Stein and Barrington 1990), suggesting that *L. ensifolia* was the maternal progenitor of the hybrid. The presence of multiple *LEAFY* alleles in $\times L.$ *Lindsaeosoria* is expected, given its hybrid origin, high chromosome numbers, and apparent polyploidy of its parental species (*L. ensifolia*, $n=88$, Manton and Sledge, 1954; *O. chinensis*, $n=48$, $n=96$, Lin, Kato, and Iwatsuki, 1990, 1994). It is likely that multiple alleles would have been observed also in the other taxa studied, if they had been sequenced using Illumina and not Sanger sequencing. Nevertheless, the tree obtained from an

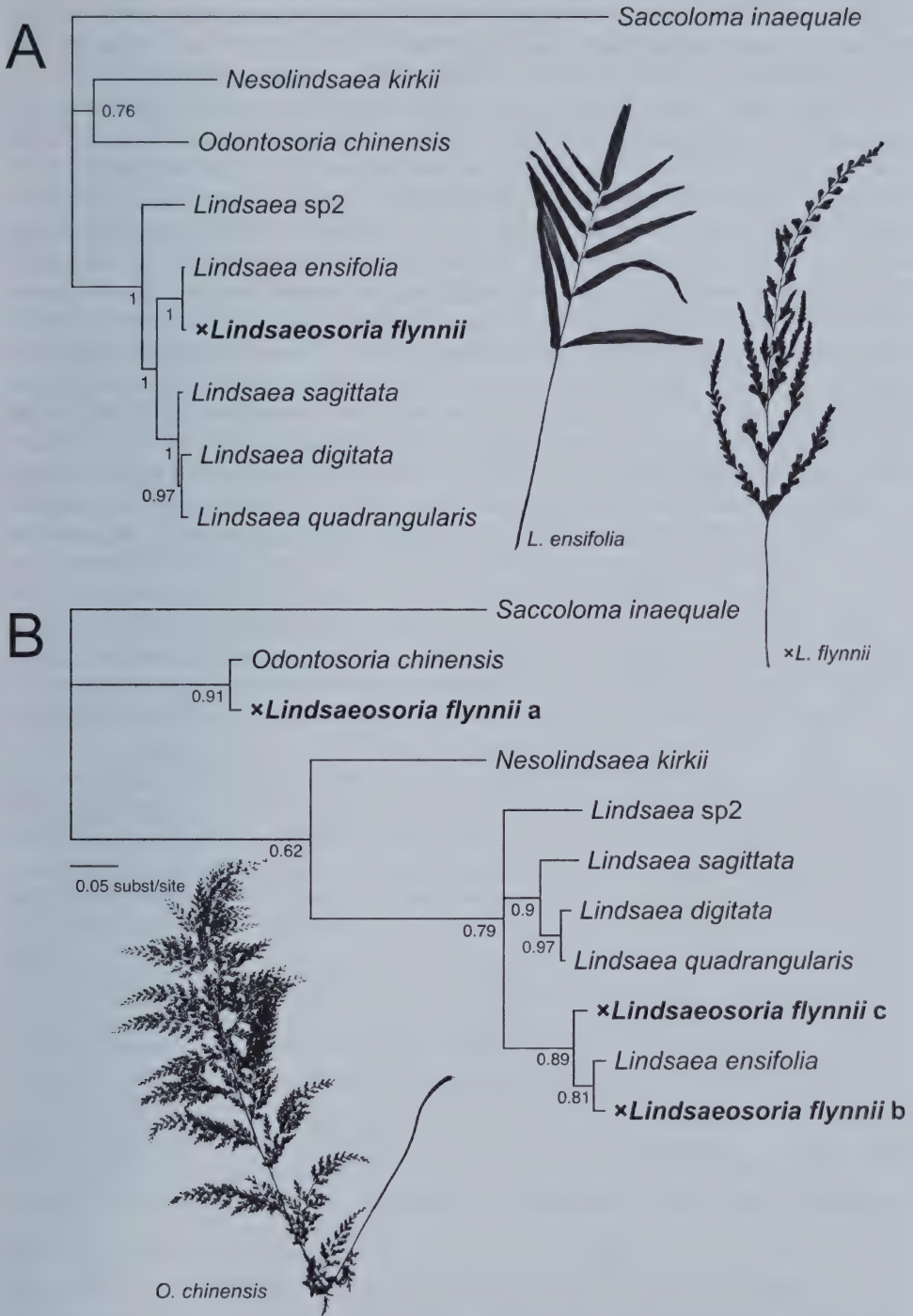


FIG. 1. Majority-rule consensus trees from Bayesian analyses with mean branch lengths across the sampled posterior trees and posterior probabilities indicated at the nodes. Both trees are in the same scale. A. Tree based on the combined analysis of plastid markers. B. Tree based on the nuclear gene *LEAFY*. The three different alleles observed in *×Lindsaeosoria flynnii* are marked with a – c.

analysis of *LEAFY* data is congruent with the current phylogenetic understanding based on plastid markers (Lehtonen *et al.*, 2010) and therefore the Sanger sequences are considered sufficient for the present purpose.

Rothfels *et al.* (2015) estimated that the parental genera of \times *Cystocarpium* diverged from each other 57.9 Ma (95% HPD 40.2–76.2 Ma) and concluded that it represented the deepest documented natural hybridization in plants or animals. \times *Lindsaeosoria* is a comparably dramatic example of natural hybridization: Lehtonen, Wahlberg, and Christenhusz (2012) estimated that *Lindsaea* and *Odontosoria* split 45.2 Ma (38.0–52.3 Ma), but the age estimates of that study were in general younger than in other studies and therefore may be underestimated. Roughly similar age estimate (46.3–52.9 Ma) was given by Schuettpelz and Pryer (2009). Regalado *et al.* (2017) dated the split somewhat older at ca. 60 Ma (30.4–98.4 Ma, depending on the approach used). Likewise, Lehtonen *et al.* (2017) estimated the *Lindsaea*-*Odontosoria* split to predate the split of *Cystopteris* and *Gymnocarpium* (estimated at 62.3 Ma, 95% HPD 35.4–93.8 Ma). In that study, however, *Odontosoria* was not resolved as monophyletic and the estimated split between *O. chinensis* and *L. ensifolia* (127.5 Ma, 95% HPD 106.5–150.9 Ma) is most likely unreasonably old. In that study the divergence between *Lindsaea* and the Neotropical *Odontosoria* was estimated to have occurred at 79.9 Ma (64.9–95.6 Ma), an estimate close to that of Testo and Sundue (2016) for the split between *Lindsaea* and *Odontosoria* at 71.1 Ma (65.0–72.0 Ma). According to the results of Testo and Sundue (2016), *Cystopteris* and *Gymnocarpium* probably represent a somewhat deeper split (77.4 Ma, 95% HPD 65.5–81.8 Ma). Given the uncertainties in molecular dating it is not possible to say which of the two hybridization events crosses an older split, but it is clear that both are dramatic examples involving lineages that have been evolutionarily separate since the Late Cretaceous or early Paleogene.

Other intergeneric fern hybrids have been described, but most of them involve genera that are no longer accepted and therefore represent intrageneric hybrids instead (Table 1). The proposed nothogenus \times *Hemionanthes* Mickel involve parental genera (*Hemionitis* L. \times *Cheilanthes* Sw.) with younger splits (Oligocene; Lehtonen *et al.*, 2017, Testo and Sundue, 2016) than \times *Cystocarpium* or \times *Lindsaeosoria*. \times *Cyclobotrya* Engels & Canestraro (*Cyclodium* C. Presl \times *Polybotrya* Humb. & Bonpl.) is a recently described nothogenus (Engels and Canestraro, 2017; Schwartzburd *et al.*, 2017) with an uncertain dating of its presumed parental genera; Lehtonen *et al.* (2017) resolved the parental genera *Cyclodium* and *Polybotrya* nested within each other with an Oligocene crown group age, whereas Testo and Sundue (2016) found the genera monophyletic with a split dated at 71.2 Ma (95% HPD 67.57–71.22 Ma). This estimate seems unreasonably old, given that Testo and Sundue (2016) estimated the split between *Polybotrya* and *Maxonia* to have occurred in the Miocene-Oligocene, and a recent study focusing on the phylogeny of the clade strongly supported *Polybotrya* as more closely related to *Cyclodium* than to *Maxonia* (Moran and Labiak, 2015). The Miocene-Oligocene time frame is in line with the estimated 23.3 Ma age for the split by Schuettpelz and Pryer (2009). However, the nothogenera \times *Polysticalpe* and \times *Dryostichum* proposed

for the hybrids between *Polystichum* and *Dryopteris* may represent the deepest fern hybrids if confirmed, with their presumed parental genera estimated to have split during the Early-Late Cretaceous or early Paleogene (Le Péchon *et al.*, 2016: 65 Ma, 95% HPD 48–82 Ma; Lehtonen *et al.*, 2017: 98.81 Ma, 95% HPD 75.6–122.5 Ma; Schuettpelez and Pryer, 2009: 67.8 Ma; Testo and Sundue, 2016: 105.67 Ma, 95% HPD 98.97–111.29 Ma).

The deep fern hybrids help to understand the genome evolution and genetic basis of the evolution of reproductive barriers and hence, fern diversification dynamics (Rothfels *et al.*, 2015). Ranker and Sundue (2015) hypothesized that ferns are less diverse than angiosperms because of slower evolution of reproductive barriers in ferns, as manifested by the deep hybrids. This hypothesis would predict that fern species are in general older than angiosperm species, a prediction that indeed seems to have support. Hedges *et al.* (2015) investigated times-to-speciation among various organisms and found that it takes on average 3.6 Ma for ferns to speciate whereas angiosperms speciate on average in 0.75 Ma. It is important to further investigate speciation times and hybridization events to better understand the evolution of reproductive barriers and the conditions under which they fail.

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APPENDIX A. LIST OF TAXA SAMPLED WITH GENBANK ACCESSION NUMBERS, REFERENCES AND VOUCHER INFORMATION.

Lindsaea digitata Lehtonen & Tuomisto, *Tuomisto 14470* (TUR), Peru: *trnL-trnF* EU146057 (Lehtonen & Tuomisto, 2007), *trnH-psbA* EU146041 (Lehtonen & Tuomisto, 2007), *rpoC1* EU146051 (Lehtonen & Tuomisto, 2007), *rps4* GU478698 (Lehtonen *et al.*, 2010), *rps4-trnS* GU478400 (Lehtonen *et al.*, 2010), *LEAFY* MG561419 (this study). ***Lindsaea ensifolia*** Sw., *Kessler 13597* (GOET), La Réunion: *trnH-psbA* GU478521 (Lehtonen *et al.*, 2010), *rpoC1* GU478606 (Lehtonen *et al.*, 2010), *rps4* GU478678 (Lehtonen *et al.*, 2010), *rps4-trnS* GU478380 (Lehtonen *et al.*, 2010), *LEAFY* MG561420 (this study). ***Lindsaea***

quadrangularis Raddi, *Jones 768* (TUR), Panama: *trnL-trnF* MG561417 (this study), *trnH-psbA* MG561403 (this study), *rpoC1* MG561408 (this study), *rps4* KJ628960 (Lehtonen *et al.*, 2015), *rps4-trnS* KJ628960 (Lehtonen *et al.*, 2015), *LEAFY* MG561421 (this study). ***Lindsaea sagittata*** Dryand., *Christenhusz 2618* (TUR), Suriname: *trnL-trnF* FJ361024 (Lehtonen *et al.*, 2010), *trnH-psbA* FJ360933 (Lehtonen *et al.*, 2010), *rpoC1* FJ360979 (Lehtonen *et al.*, 2010), *rps4* GU478688 (Lehtonen *et al.*, 2010), *rps4-trnS* GU478390 (Lehtonen *et al.*, 2010), *LEAFY* KM114113 (this study). ***Lindsaea sp.2***, *Kluge 7230* (GOET), Sulawesi: *trnL-trnF* FJ361022 (Lehtonen *et al.*, 2010), *trnH-psbA* FJ360931 (Lehtonen *et al.*, 2010), *rpoC1* FJ360977 (Lehtonen *et al.*, 2010), *rps4* GU478664 (Lehtonen *et al.*, 2010), *rps4-trnS* GU478366 (Lehtonen *et al.*, 2010), *LEAFY* MG561422 (this study). ***Nesolindsaea kirkii*** (Baker) Lehtonen & Christenh., *Christenhusz 5880* (H), Seychelles: *trnL-trnF* MG561416 (this study), *trnH-psbA* MG561404 (this study), *rpoC1* HQ157323 (Lehtonen *et al.*, 2012), *rps4* HQ157327 (Lehtonen *et al.*, 2012), *rps4-trnS* MG561411 (this study), *LEAFY* KM114115 (this study). ***Odontosoria chinensis*** (L.) J.Sm., *Kessler 13542* (GOET), La Réunion: *trnL-trnF* MG561415 (this study), *trnH-psbA* MG561405 (this study), *rpoC1* HQ157313 (Lehtonen *et al.*, 2012), *rps4* HQ157328 (Lehtonen *et al.*, 2012), *rps4-trnS* MG561412 (this study), *LEAFY* KM114116 (this study). ***Saccoloma inaequale*** (Kunze) Mett., *Jones 1019* (TUR), Panama: *trnL-trnF* MG561418 (this study), *trnH-psbA* MG561402 (this study), *rpoC1* MG561407 (this study), *rps4* MG561410 (this study), *rps4-trnS* MG561410 (this study), *LEAFY* KM114112 (this study). ***×Lindsaeosoria flynnii*** W.H.Wagner, *Flynn 2351* (Z), Hawaii: *trnL-trnF* MG561414 (this study), *trnH-psbA* MG561406 (this study), *rpoC1* MG561409 (this study), *rps4* MG561413 (this study), *rps4-trnS* MG561413 (this study), *LEAFY* MG561423, MG561424, MG561425 (this study).

Upright Fronds of *Dryopteris intermedia* Suffer Frost Damage and Breakage during Winter

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ABSTRACT.—The most abundant fern species in northeast United States forests are wintergreen. These ferns keep their fronds for one year before replacing them in the spring. The wintergreen fronds soften at their base in fall and survive the winter under snow, allowing vernal photosynthesis and providing an energetic benefit to the plant. This study addressed whether or not the softening of the stipe is critical to the evolutionary adaptiveness of the wintergreen leaf habit. I kept the fronds of 12 *Dryopteris intermedia* plants upright through the winter of 2015–2016 and left those of 12 other plants prostrate as controls. In the spring of 2016, a greater proportion of the frond surface area had frost damage on the upright fronds than on the control plants, and a greater proportion of the fronds were broken in the upright plants than in the control plants. The softening of the stipe was probably a critical step in the evolution of the wintergreen habit in *D. intermedia*.

KEY WORDS.—deciduous forest, Dryopteridaceae, fern, herbaceous layer, understory.

Leaf habit encompasses the timing of leaf production, the longevity of the leaves, and the timing of leaf senescence (Husk and Warton, 2007; Kikuzawa, 1991; Ordoñez et al., 2010). Understory herbaceous plants of forests include species that are spring ephemeral (Nault and Gagnon, 1993; Yoshie and Fukuda, 1994), deciduous (Oikawa et al., 2004; Rothstein and Zak, 2001), evergrowing (Monasterio and Sarmiento, 1976), seasonalgreen (Tessier, 2004), wintergreen (Flinn, 2006; Uemura, 1994), semi-evergreen (Bauer, Gallmetzer, and Sato, 1991), and evergreen (Hughes, Neufeld, and Burkey, 2005). In forests of the northeast United States and eastern Canada, the most abundant ferns are wintergreen (Flinn, 2007; Montgomery, 1976; Siccama, Bormann, and Likens, 1970; Singleton et al., 2001), producing a set of fronds in the spring, retaining them through the summer, fall, and winter, and replacing them with a new set the following spring (Damascos et al., 2005; Tessier, 2008). Representative species include *Dryopteris intermedia* (Willd.) A. Gray, *D. marginalis* (L.) A. Gray, and *Polystichum acrostichoides* (Michx.) Schott.

To accomplish the prostrate nature that the fronds have under winter snow, the base of the stipe softens thereby lowering the frond to the level of the leaf litter (Noodén and Wagner, 1997). These fronds photosynthesize through the fall after canopy leaves have senesced and in spring prior to canopy leaf out, contributing 29% of annual carbon gain in *D. intermedia* (Goldblum and Kwit, 2012, 2013). The vernal fixed carbon is moved back to the plant, allowing these fronds to provide an energetic benefit (Minoletti and Boerner, 1993; Tessier, 2001; Tessier and Bornn, 2007; Van Buskirk and Edwards, 1995). Therefore, the wintergreen leaf habit generates a meaningful carbon enrichment compared to the deciduous leaf habit in which plants lose their leaves in

the fall (Killingbeck et al., 2002), most likely increasing the frequency of the wintergreen species in northeastern forests (García, Picó, and Ehrlén, 2008; Reich et al., 2003). As a result, determining how this leaf habit developed evolutionarily in northeastern fern species is important to understanding their abundance and distribution in ecosystems.

This study addressed a question related to the evolution of the wintergreen leaf habit in ferns of the northeastern United States. Could ferns have evolved to be wintergreen without the softening of the stipe in the fall? I hypothesized that being prostrate protects fronds from damage during winter and predicted that fronds held upright through winter would be damaged by frost and/or broken in spring.

MATERIALS AND METHODS

To assess the importance of fronds becoming prostrate prior to winter, I forced fronds of *Dryopteris intermedia* to remain upright through winter in tomato cages. A 60 m transect was constructed through a second growth hardwood forest near Delhi, NY USA (42° 14' 53" N Lat., 74° 56' 06" W Long.) in November 2015 (Tessier, 2017). The site has a northeast aspect and steep slope. At 5 m intervals, the two *D. intermedia* plants nearest the transect line were added to the study. The 60 m transect encompassed a range of microtopography representative of the site (rocky, pit, exposed, etc.), and the pairing of the treatments helped to limit the impact of this variation in microtopography on the experiment. Adding additional sites to assess the effect of other environmental variables was beyond the scope of the project, and would have added more background noise to the data. One plant per pairing had its fronds raised upright and held in place within a circular, 50 cm tall tomato cage. The other plant (control) had its fronds physically raised upright (as if to be placed in a tomato cage) and then returned prostrate. This raising and lowering controlled for any damage suffered by the act of raising the fronds into the tomato cages. Tomato cages were not temporarily installed on the control plants, because removing them would have added another experimental variable (damage from cage removal) that was not present in the treatment plants. Thus, the only variable that was different between the treatment and control plants was the fronds staying upright through winter in the treatment plants. Twelve replicates were established in this fashion. Which plant (closest vs. second closest) became the upright treatment and which became the control alternated at successive 5 m locations.

Monthly during the winter (December through March), the plants were visited to move any fronds that had come loose from the cages back to an upright position. Snow depth and temperature measurements were taken at 15 points at 2 m intervals across a 30 m transect that was parallel to the transect in which the plants in this study were located. The winter of 2015–2016 had relatively little snow, with average mid-month snow depth at the site of 0 cm in December, 4.2 cm in January, 1.5 cm in February, and 0 cm in March

(Tessier, unpublished data). The average temperature of the air at those dates was 7.9 °C in December, -0.3 °C in January, -7.2 °C in February, and 5.5 °C in March (Tessier, unpublished data). Such winters are expected to become increasingly common in the northeast United States (Groffman et al., 2012).

In April 2016, the amount of damaged, undamaged, and total surface area (leaf cover *sensu* Fehmi, 2010) on all of the fronds was measured to the nearest 100 cm². Damaged frond area was identified by its brown, dry surface, which is indicative of frost damage. These measurements were taken in the field by placing a 100 cm² grid over the fronds and measuring the damaged and undamaged area. Cells without frond material were not included in the count. Dividing the damaged area by the total frond area yielded the proportion of frond area that was damaged. Also, the number of fronds with a broken or unbroken rachis or stipe was counted on each plant. For the plants in this study, there was an average of 6.21 fronds per plant. Fronds in *D. intermedia* range from 30–80 cm in length in the northeast United States (Gleason and Cronquist, 1991).

The proportion of surface area damaged and the proportion of fronds with broken rachises or stipes were each compared between the upright and control treatments using a Mann-Whitney test at $\alpha = 0.05$ with a sample size of 12 in Minitab version 17 (Minitab, Inc., State College, Pennsylvania, USA). A non-parametric test was used due to lack of normality in the data, which could not be corrected by transformation.

RESULTS

Significantly more frond area was frost damaged in the upright fronds than in the prostrate fronds of *D. intermedia* (Fig. 1; $W = 222.0$, $df = 1$, $P < 0.0001$). On average, just over 60% of the frond area was damaged in the upright fronds compared to just over 20% in the prostrate fronds (Fig. 1). A significantly greater proportion of rachises or stipes was broken in the upright fronds than in the prostrate fronds of *D. intermedia* (Fig. 2; $W = 214.5$, $df = 1$, $P = 0.0002$). On average, over 60% of the rachises or stipes in upright fronds was broken compared to 5% in the prostrate fronds (Fig. 2).

DISCUSSION

Without the ability to make the overwintering fronds prostrate, the adaptiveness of being wintergreen would suffer a dramatic loss (Figs. 1 and 2). This result supports the hypothesis that the prostrate position protects overwintering fronds from damage. Losing over 60% of both their photosynthetic surface area to frost damage (Fig. 1) and rachises or stipes to breakage (Fig. 2) would severely limit the utility of keeping fronds through the winter. Therefore, the evolution of the ability to soften the base of the stipe and allow fronds to be prostrate (Noodén and Wagner, 1997) was probably a critical and early adaption in the evolution of the wintergreen leaf habit. Smaller herbaceous species with the wintergreen leaf habit, such as *Cornus canadensis*

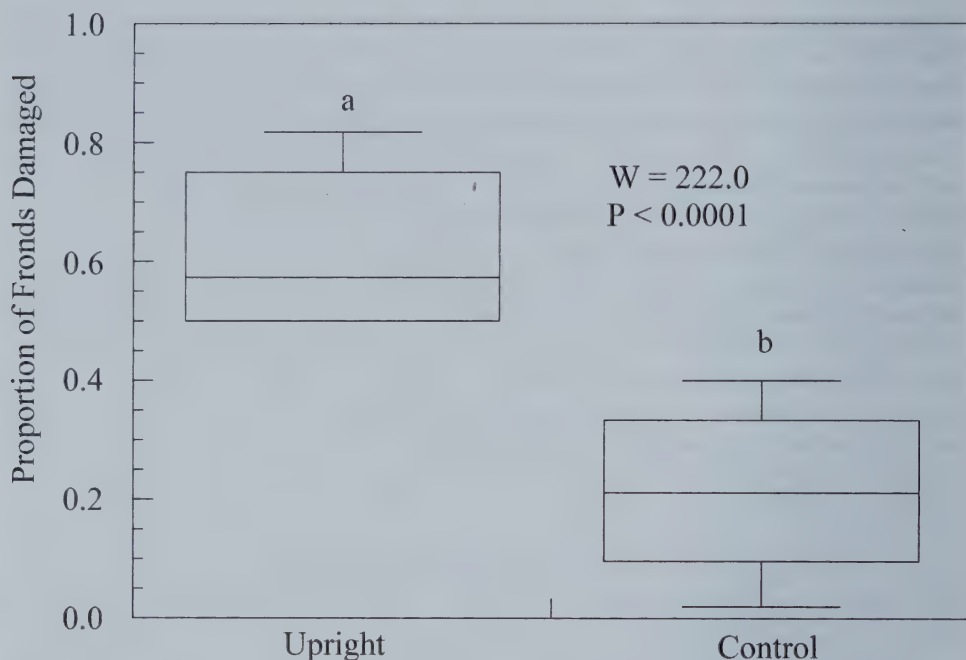


FIG. 1. Proportion of *Dryopteris intermedia* (Willd.) A. Gray fronds with frost damage between plants with fronds held upright versus those with fronds left prostrate through the winter of 2015–2016 in a second growth hardwood forest in Delhi, NY USA. The center line is the median, the top of box is the upper 75%, the bottom of box is the bottom 25%, and the whiskers are the 10% and 90% marks. Treatments with a different letter are significantly different at $\alpha = 0.05$ based on a Mann-Whitney test.

L. (Landhäuser, Stadt, and Lieffers, 1997), *Anemone americana* (DC.) H. Hara (Beatley, 1956), and *Tiarella cordifolia* L. (Rothstein and Zak, 2001) could avoid the need for a softened and prostrate stem by staying beneath protective snow owing to their diminutive stature. Therefore, the adaptiveness of a softened stem base most likely only arose in more upright and tall species, such as the wintergreen ferns of the northeast United States.

Ferns and other plants are susceptible to frost damage, particularly as climate change leads to late frosts and unusual precipitation (Augspurger, 2009; Tessier, 2014; Wheeler et al., 2016). Variability in frost tolerance among species has implications for those species' habitat and range (Bannister, 1984; Ealson et al., 2013; Gusta and Wisniewski, 2013; Lüttge, 2013). Adaptations to frost, such as making fronds prostrate, allow plants to expand their photosynthetic season throughout their range where conditions permit (Bannister and Fagan, 1989; Taschler and Neuner, 2004). Therefore, developing a softened base to the stipe will have provided a significant photosynthetic advantage over species without this hinge in snow-covered areas (Killingbeck et al., 2002).

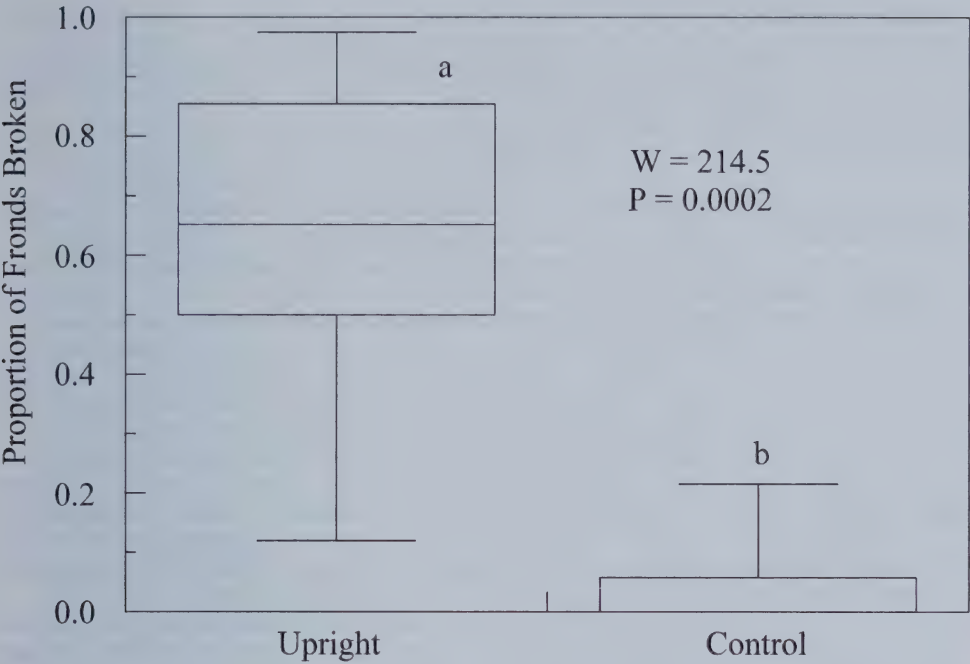


FIG. 2. Proportion of *Dryopteris intermedia* (Willd.) A. Gray fronds with broken rachises / stipes between plants with fronds held upright versus those with fronds left prostrate through the winter of 2015–2016 in a second growth hardwood forest in Delhi, NY USA. The center line is the median, the top of box is the upper 75%, the bottom of box is the bottom 25%, and the whiskers are the 10% and 90% marks. Treatments with a different letter are significantly different at $\alpha = 0.05$ based on a Mann-Whitney test.

These results lead to a two new questions. Many other species of *Dryopteris* and *Polystichum* that live in the northeast United States are wintergreen, including *D. marginalis* (L.) A. Gray, *D. clintoniana* (D.C. Eaton) Dowell, *D. fragrans* (L.) Schott, *P. acrostichoides* (Michx.) Schott, *P. braunii* (Spenn.) Fée, and *P. lonchitis* (L.) Roth (Gleason and Cronquist, 1991). Based on the results of this study, one new question is whether or not all species with softened stipe-bases would suffer similar damage if kept upright through winter. The second question is: would the breaking of rachises or stipes prevent the movement of carbon and other resources from the old fronds back to the persisting plant components? If the breakage does prevent such movement, there is further evidence that the softening of the stipe was a critical adaptation for the support of the wintergreen leaf habit.

In conclusion, the ability to make intact fronds prostrate to the leaf litter appears to have been a critical adaptation in the evolutionary development of the wintergreen leaf habit in *Dryopteris intermedia*. New questions relate to the ubiquity of this pattern of damage in upright fronds and movement of resources from broken fronds to the rest of the plant with impacts on the timing and rate of development of new fronds.

ACKNOWLEDGMENTS

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Novelties in Costa Rican *Pityrogramma* (Pteridaceae): A New Species and a New Hybrid from the Osa Peninsula

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ABSTRACT.—*Pityrogramma hirsuta* Testo is described from a secondary lowland rain forest on the Osa Peninsula of Costa Rica. It is distinguished from all other Costa Rican *Pityrogramma* species by a combination of once-pinnate-pinnatifid laminae, abundant whitish hairs on the petiole, rachis, and laminar surface, and complete lack of farina on the abaxial laminar surface. A hybrid between this species and *Pityrogramma calomelanos* (L.) Link, *Pityrogramma* ×*watkinsii* Testo is also described. It is morphologically intermediate to its progenitors and is characterized by an indument of whitish hairs on the petiole, rachis, and laminar surface, and sparse white farina on the abaxial surface of the lamina. The new species and hybrid are each known only from the type collections. Relationships to other species of *Pityrogramma* are discussed, and a key to Central American *Pityrogramma* is presented.

KEY WORDS.—Central America, Neotropics, new species, *Pityrogramma*, taxonomy

While studying the fern flora of Costa Rica's Osa Peninsula in January, 2016, I encountered several unusual plants that belonged to the genus *Pityrogramma* but did not seem closely allied to any known Central American species. Closer examination of collections made from these plants indicated that they represented two taxa: one fertile species, and a sterile hybrid derived from a cross between that species and another congener. Comparison of this material to *Pityrogramma* collections at CR, HUA, HULE, MEXU, NY, and VT, as well as study of images of types and other relevant material indicated that both taxa are new to science. They are described here, starting with the species.

Pityrogramma hirsuta Testo, sp. nov. **TYPE.**—COSTA RICA. Provincia Puntarenas: Osa Peninsula, Dos Brazos de Río Tigre, Bolita Rainforest Reserve, Valle Frijol trail, clearing in secondary forest dominated by *Heliconia*. 8°30'52"N 83°24'21"W, 250 m, 6 February 2016, W.L. Testo 1015 (holotype: VT!, isotypes: CR!, NY!, UC!) (Figs. 1, 2).

DESCRIPTION.—*Rhizome* 0.5–1 cm in diameter, erect; *rhizome scales* 3–6 × 0.3–0.5 mm, linear-lanceolate, golden-brown; *fronds* 40–70 × 6–18 cm; *petiole* 10–30 cm long, grooved slightly adaxially, dark brown, with abundant scales near base, spreading, catenate whitish hairs throughout; *rachis* grooved adaxially, with hairs like those of the petiole; *lamina* 25–50 × 6–18 cm, narrowly lanceolate to elliptic, 1-pinnate-pinnatisect, slightly reduced at base, apex

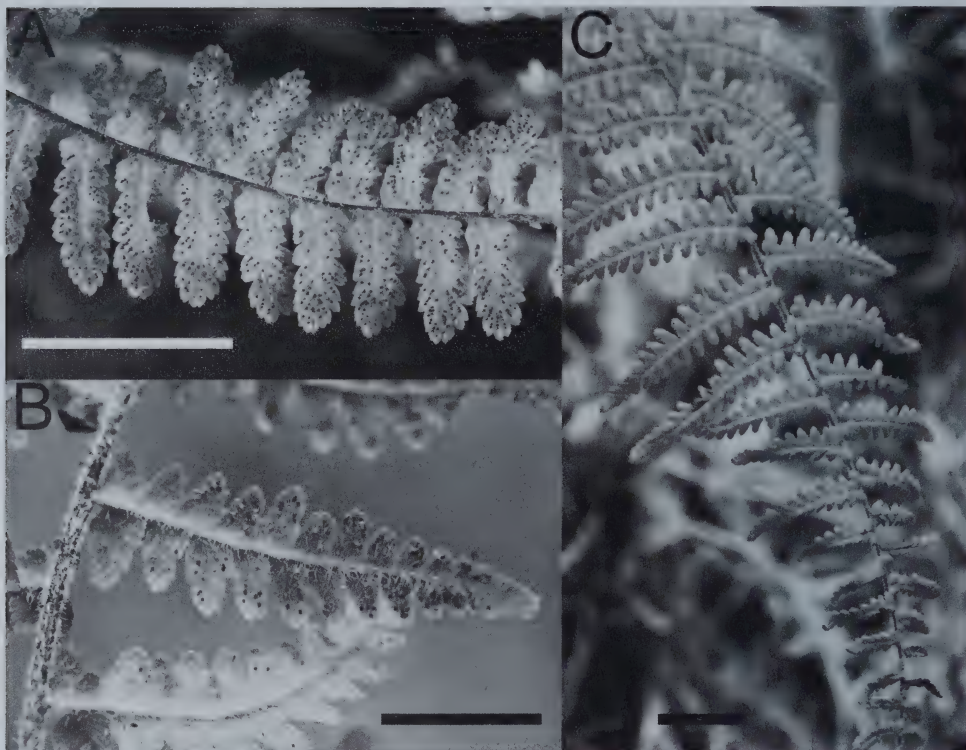


FIG. 1. Details of *Pityrogramma hirsuta*. A. Abaxial surface of medial pinna. B. Abaxial surface of rachis and distal pinna. C. Leaf apex. Scale bar = 2.5 cm.

gradually tapered; *pinnae* $5-9 \times 0.5-1.2$ cm, 10–17 free pairs, divided nearly or completely to costa, pinnules rounded; *laminae* herbaceous, with catenate hairs like those of leaf axes abaxially, glabrous or nearly so adaxially; *veins* free, running to leaf margin, with short light-brownish hairs abaxially; *sori* scattered along veins; *sporangia* glabrous; *spores* $47-53 \mu\text{m}$, reddish-brown, trilete.

ETYMOLOGY.—The specific epithet refers to the pubescent leaf axes and laminar surfaces of this species.

DISCUSSION.—This new species is known only from a single collection made at the Bolita Rainforest Reserve near Dos Brazos del Río Tigre, on Costa Rica's Osa Peninsula. Thorough study of the extensive *Pityrogramma* collections at the National Museum of Costa Rica (CR) failed to reveal additional specimens of *P. hirsuta*, suggesting that it has not been previously collected in Costa Rica. This species was abundant along a trail near disturbed secondary forest, and occurred with *Heliconia* spp., *Solanum rovirosanum* Donn. Sm., *Pityrogramma calomelanos* (L.) Link, and *Tectaria rufovillosa* (Rosenst.) C. Chr.



FIG. 2. Holotype of *Pityrogramma hirsuta* (Testo 1015, VT).

Among Central American *Pityrogramma*, only two other species possess pubescent, non-farinose abaxial laminar surfaces: *Pityrogramma ferruginea* (Kunze) Maxon and *P. ochracea* (C. Presl) Domin. Both species can be distinguished from *P. hirsuta* by their reddish-brown (vs. whitish) indument, as well as the distribution of hairs on the leaf. In *P. hirsuta*, hairs are distributed evenly and at a moderate density across the leaf axes and abaxial surface of the lamina. In *P. ferruginea*, the rachis, costae, and abaxial surface of the lamina are so densely covered in hairs that they are mostly or completely obscured. Conversely, the hairs of *P. ochracea* are almost entirely restricted to the laminar surface; the rachis and petiole are nearly glabrous.

Critical to this work was the study of *Pityrogramma* from beyond Central America, especially given the phytogeographical affinities between the Osa Peninsula and northwestern South America (Cornejo et al., 2012). Among the remaining species of *Pityrogramma*, only the Andean *Pityrogramma jamesonii* (Baker) Domin is pubescent and completely non-farinose. This species can be readily distinguished from *P. hirsuta* by its broadly lanceolate lamina, glabrous or nearly glabrous leaf axes, and acute pinnule apices. Two other species from the northern Andes, *Pityrogramma lehmannii* (Hieron.) R.M. Tryon and *P. opalescens* Sundue, also possess hairs on the abaxial surface of the lamina; however, these hairs differ from those of other *Pityrogramma* species in that they bear farina-producing glands at their apex (Sundue, 2011). These species are further distinct from other *Pityrogramma* species in that their lamina is merely pinnatisect; further study is needed to confirm the phylogenetic placement of these anomalous taxa.

Pityrogramma hirsuta bears a striking resemblance to *P. boucheana* (A. Braun) Domin, an enigmatic taxon described (as *Gymnogramme boucheana*) by Alexander Braun (Braun, 1854). Braun described *P. boucheana* from a single plant at the Berlin Botanical Garden that appeared in a planting of *P. ferruginea*, and he hypothesized it was a hybrid between that species and *P. chrysophylla*. Domin (1929) transferred *G. boucheana* the species to *Pityrogramma* and agreed with the parentage proposed by Braun, citing its pubescence and divaricate pinnule attachment as evidence that *P. ferruginea* was one parent and the presence of yellow farina to support the involvement of *P. chrysophylla* as the other. Examination of a high-resolution image of the holotype of *P. boucheana* at K [A. Braun s.n.; barcode: K001057896] confirmed the presence of sparse yellow farina on the lamina and showed that the hairs present were reddish-brown, in agreement with the proposed hybrid origin and confirming the distinctness of *P. hirsuta* from *P. boucheana*. It is unsurprising that *P. boucheana* is only known from cultivation, as *P. ferruginea* is Central American (disjunct in central Peru) and *P. chrysophylla* is restricted to Puerto Rico and the Lesser Antilles (Tryon, 1962).

Given that farina color and presence can sometimes vary within some species of *Pityrogramma* (Tryon, 1962), the possibility that *P. hirsuta* may simply be an aberrant variant of a typically farinose *Pityrogramma* species

warrants consideration. Only three such species are known from the Pacific lowlands of Costa Rica: *Pityrogramma calomelanos*, *P. dealbata* (C. Presl) Domin, and *P. trifoliata* (L.) R.M. Tryon. The latter two species can be readily distinguished from *P. hirsuta* based on leaf shape alone: *P. dealbata* has a broadly deltate lamina, whereas *P. trifoliata* has lanceolate leaves with entire or 2–7-foliolate pinnae that are rotated out of the plane of the leaf. *Pityrogramma calomelanos* is more similar to *P. hirsuta* than the aforementioned two species, but differs by typically having twice-pinnate-pinnatifid leaves with acute, ascending pinnules (vs. once-pinnate-pinnatisect leaves with obtuse and divaricately arranged pinnules in *P. hirsuta*). These differences were evident when the two species were observed co-occurring at the type locality of *P. hirsuta*. Additional evidence that *P. hirsuta* is not merely an aberrant form of *P. calomelanos* is the existence of an abortive-spored hybrid between the two species. A single plant of this morphologically intermediate hybrid was observed growing with *P. hirsuta* and *P. calomelanos*; it is described here.

Pityrogramma* × *watkinsii Testo, hyb. nov. TYPE.—COSTA RICA. Provincia Puntarenas: Osa Peninsula, Dos Brazos de Río Tigre, Bolita Rainforest Reserve, Valle Frijol trail, clearing in secondary forest dominated by *Heliconia*. 8°30'52"N 83°24'21"W, 250 m, 6 February 2016, W.L. Testo 1016 (holotype: VT!, isotype: CR!) (Fig. 3).

DESCRIPTION.—*Rhizome* 1–2.5 cm in diameter, erect; *rhizome scales* 4–8 × 0.3–0.5 mm, linear-lanceolate, golden-brown; *fronds* 65–80 × 20–36 cm; *petiole* 28–34 cm long, grooved slightly adaxially, dark brown, with abundant scales near base, spreading, catenate whitish hairs sparse; *rachis* grooved adaxially, with sparse hairs like those of the petiole, *lamina* 35–50 × 20–36 cm, lanceolate, 2-pinnate-pinnatifid, broadest near base, apex gradually tapered; *pinnae* 10–15 × 1.0–2.2 cm, 19–27 free pairs, divided completely to costa, pinnules acute, deeply lobed; *laminae* herbaceous, with sparse white farina and sparse catenate hairs like those of leaf axes abaxially, glabrous or nearly so adaxially; *veins* free, running to leaf margin, with short light-brownish hairs abaxially; *sori* scattered along veins; *sporangia* glabrous, often malformed; *spores* highly irregular, reddish-brown, tetrahedral.

ETYMOLOGY.—The epithet honors fern ecologist James “Eddie” Watkins, a close friend and mentor who has encouraged my studies of Costa Rican ferns, especially *Pityrogramma*.

DISCUSSION.—*Pityrogramma* × *watkinsii* is a hybrid between *Pityrogramma hirsuta* and *Pityrogramma calomelanos*, and is morphologically intermediate between these species. It is recognizable by a combination of the following characters: sparse white farina on the abaxial surface of the lamina, scattered whitish hairs on the lamina and leaf axes, and irregular spores. It is only known from the type collection.



FIG. 3. Holotype of *Pityrogramma watkinsii* (Testo 1016, VT).

To aid in identification of Central American *Pityrogramma* species, the following key is presented:

KEY TO CENTRAL AMERICAN *PITYROGRAMMA* SPECIES

1. Distal pinnae entire, pinnae twisted out of plane of leaf, proximal pinnae even-sided or broadest near middle *P. trifoliata*
1. Distal pinnae lobed or more divided, pinnae not twisted, proximal pinnae broadest near base 2.
2. Abaxial surface of lamina pubescent or glabrous, not farinose 3.
3. Lamina 4–5-pinnate, abaxial surface of lamina glabrous *P. pearcei*
3. Lamina to 3-pinnate-pinnatifid, abaxial surface of lamina pubescent 4.
4. Hairs on abaxial surface of lamina and rachis whitish *P. hirsuta*
4. Hairs on abaxial surface of lamina and rachis reddish-brown 5.
5. Rachis obscured by dense hairs, pinnules sessile *P. ferruginea*
5. Rachis visible, pinnules stalked *P. ochracea*
2. Abaxial surface of lamina farinose 6.
6. Proximal pinnae elongated basiscopically 7.
7. Rachis tan or straw-colored, farina yellow *P. chrysoconia*
7. Rachis castaneous or blackish, farina white. *P. ebenea*
6. Proximal pinnae not elongated basiscopically 8.
8. Farina white, typically elevations < 1000 m *P. calomelanos*
8. Farina yellow, typically elevations > 1000 m .. *P. austroamericana*

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